

## 245 - A Highly Sensitive Glucose Electrode Using Glucose Oxidase Collagen Film \*

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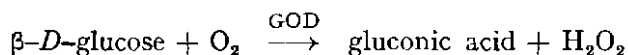
### Summary

Industrially reconstituted collagen films have shown excellent properties for  $\beta$ -D-glucose oxidase coupling. Associated with a platinum anode for amperometric detection of hydrogen peroxide, these enzymatic films form a very simple and easy to handle glucose electrode; this device presents a very high sensitivity (*ca.*  $10^{-8}$  M) giving responses proportional to glucose concentration over 5 orders of magnitude.

### Introduction

The association of an enzyme in a soluble form with an electrochemical sensor was first reported by CLARK and LYONS, in 1962.<sup>1</sup> Since this date, the obtainment of carrier-bound enzymes has permitted the design of enzyme electrodes. In most cases, enzymes are trapped in gels surrounding the sensor<sup>2</sup> leading to systems which are difficult to handle.

In the present work, an enzymatic membrane was prepared from reconstituted calf skin collagen after acyl-azide activation and a coupling process giving a surface binding of enzymes of different classes.<sup>3-5</sup> A glucose electrode using  $\beta$ -D-glucose oxidase (GOD) membranes was developed, using an amperometric method with a platinum electrode to detect hydrogen peroxide, which is a product of the enzymatic oxidation of glucose according to the reaction:



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The potential was fixed at +650 mV *vs* Ag|AgCl, KCl 0.1 *M* and the anodic current was recorded. A stationary response was then obtained allowing the measurement of very low glucose concentrations. In a more sophisticated device, a second electrode involving a non-enzymatic membrane was used to compensate for the detection of other electroactive molecules and to enhance the selectivity of the glucose electrode.<sup>6,7</sup>

## Experimental

### *Instrumentation*

The glucose electrode consisted of a modified gas electrode in which the pH detector was replaced by a platinum disk and the usual teflon film by a collagen membrane. In a differential device, electrode 1 was mounted with a  $\beta$ -D-glucose oxidase collagen membrane and electrode 2 with a non-enzymatic one.

Electronics were made by SOLEA TACUSSEL: current outputs of both working electrodes were first subtracted (Deltapol) and then twice differentiated (Derivol) with a time-base of one second (GSTP); thus different current *vs* time curves were available and usually recorded after a glucose pulse (SOLEA TACUSSEL EPL 2 with TV 11 GD plug-in unit, and three traces LINEAR 395 recorders).

Unless otherwise mentioned, the temperature of the solutions were carefully thermostated to  $30.0 \pm 0.1$  °C (COLORA cryothermostat WK 5 DS).

### *Solutions and reagents*

Insoluble films of highly polymerized reconstituted collagen (20 cm wide) were a gift of the Centre Technique du Cuir, Lyon (France); their thickness is about 0.1 mm in a dry state and 0.3–0.5 mm when swollen. They do not need to be tanned and can be stored several years without damage.<sup>3-5</sup>

Unless otherwise mentioned, all chemicals were reagent grade. The stock solutions of 0.1 *M* glucose were allowed to mutarotate at room temperature at least 3 hours before using and were stored at 4 °C. Both electrodes were filled with and dipped into 0.2 *M* acetate buffer, 0.1 *M* KCl solutions, pH 5.6.

### *Glucose oxidase binding on collagen membranes*

The mild general acyl azide procedure for collagen membranes activation was used<sup>3,4</sup> followed by the enzyme coupling.

Carboxyls were first esterified by immersion of crude membranes in a methanol/0.2 *M* hydrochloric acid solution for at least 72 hours, then treated overnight by 1 % hydrazine and soaked at 4 °C for 3 minutes in 0.5 *M* NaNO<sub>2</sub>—0.3 *M* HCl mixture just before coupling. Thorough washings were performed between each step and at the end of the

activation process avoiding contact between reagents and enzyme solutions.

Activated films of  $2 \times 1.5 \text{ cm}^2$  were dipped (2 hours at  $4^\circ\text{C}$ ) into  $1.5 \text{ cm}^3$  of borate buffer at pH 9 containing 2.5 mg  $\beta$ -D-glucose oxydase (GOD, E.C. 1.1.3.4, BOEHRINGER lyophilizate, grade I). Excess of soluble glucose oxydase was washed away for about 100 minutes in  $1 \text{ M}$  KCl and surface activity was in the range  $30\text{--}60 \text{ nmol. min}^{-1} \text{ cm}^{-2}$ . Enzyme collagen membranes were stored in  $0.2 \text{ M}$  acetate buffer,  $0.1 \text{ M}$  KCl (pH 5.6).

#### Procedure

Both electrodes were allowed to equilibrate in the buffer solution for 15 to 30 minutes after stepping the potential of the platinum disks to  $+650 \text{ mV vs Ag|AgCl, } 0.1 \text{ M KCl}$ . This potential corresponds to a diffusion-limited current for  $\text{H}_2\text{O}_2$  oxidation. Calibrations were performed by successive microadditions ( $10$  to  $50 \text{ mm}^3$ ) of stock solutions of  $10^{-6}$  to  $10^{-1} \text{ M}$  glucose to  $10$  to  $20 \text{ cm}^3$  of buffer. The stationary response was the variation of the steady state values of  $I_1\text{--}I_2$  when a sample of a glucose containing solution was added and the dynamic response was the height of the peak of the first derivative  $d(I_1\text{--}I_2)/dt$ . When successive additions were performed in the same solution, a current offset was used.

#### Results and discussion

When the enzyme electrode is immersed in a medium in which a pulse of glucose is added a steady state takes place after 2–3 minutes, as shown on Fig. 1, and the value of the anodic current reaches a plateau. The variation of intensity is directly dependent on the glucose concentration in the assay: this is the *stationary response*. On the other hand, the *dynamic response* is measured by the height of the peak obtained after 30–50 s by recording the first derivative of this current.

The lowest glucose concentration detected under these conditions is less than  $10^{-8} \text{ M}$  (Fig. 2). For values higher than  $10^{-3} \text{ M}$ , the responses become independent of the glucose concentration. The concentrations, which can be determined, range between *ca.*  $10^{-8}$  and  $10^{-2} \text{ M}$  *i.e.* over 6 orders of magnitude and the linearity of the calibration curve is obtained between  $(3\text{--}5) \times 10^{-8}$  and  $(3\text{--}5) \times 10^{-3} \text{ M}$  *i.e.* over 5 orders of magnitude (Fig. 2).

In a typical experiment, the calibration curves remained linear even after 40 hours operation at  $30^\circ\text{C}$  and 250 days storage at  $4^\circ\text{C}$ , allowing accurate repeated determinations for 160 micro-assays tested. However, a daily calibration was necessary, because of the slight decreasing of the calibration curve slope: *ca.* 20 % after 20 hours operation (cumulated time of measurements) and 20–50 % after 40 hours. The accuracy of glucose determinations during a set of experiments was tested by successive additions of the same aliquot of glucose (Fig. 1);

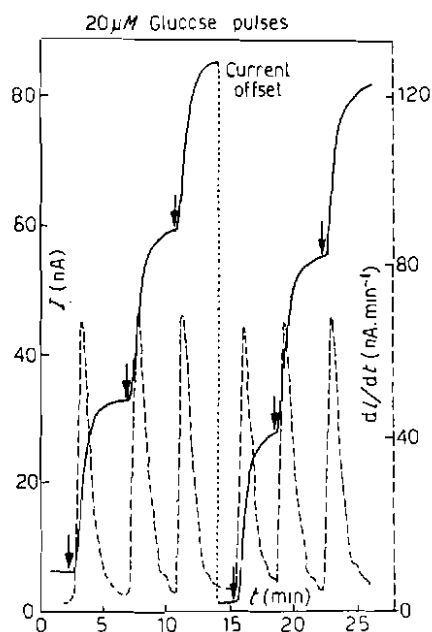


Fig. 1.

Electrode responses when successive  $20\ \mu\text{M}$  glucose pulses are added to the buffer solution. Addition of  $40\ \text{mm}^3$  ( $\mu\text{l}$ ) aliquots of  $10\ \text{mM}$  glucose solution into  $20\ \text{cm}^3$  of  $0.2\ \text{M}$  acetate buffer,  $0.1\ \text{M}$  KCl (pH 5.6) solution. (—) direct current giving stationary responses, (---) first derivative of this current giving dynamic responses.

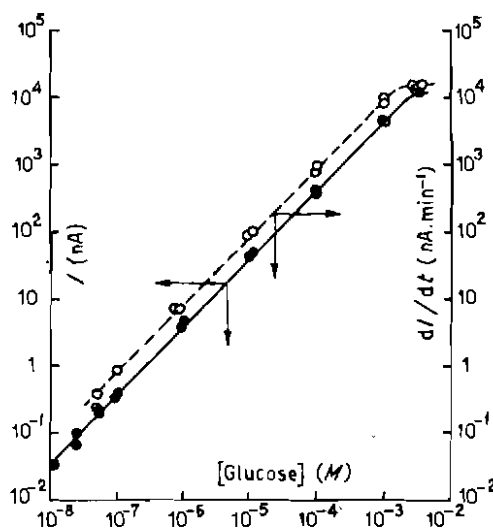


Fig. 2.

Calibration curves of the glucose electrode. (—) stationary response; (---) dynamic response.

for 13 successive additions of  $10^{-5}\ \text{M}$  glucose, the standard deviation from the mean is usually lower than 2 %.

Glucose oxidase itself is very selective for  $\beta\text{-D-glucose}$ ; thus enzymatic electrode 1 presents a high selectivity for glucose compared

with usual sugars : selectivity ratios are higher than 2 000 / 1 for fructose, lactose and sucrose. As other species may diffuse through collagen membranes and may be oxidized on platinum at +0.94 V (N.H.E.), the use of a compensating non enzymatic electrode 2 eliminates possible interferences of species such as ascorbate, urate, tyrosine or hydrogen peroxide; the selectivity ratio for hydrogen peroxide ranges between 80/1 and 250/1 depending upon experimental parameters (accuracy of the balance of both electrodes and GOD activity of the film).

The use of non-enzymatic electrode 2 is specifically of greatest interest for blood glycemia determinations,  $I_2$  usually reaching 10–50 % of  $I_1$ – $I_2$ . Fig. 3 presents the typical analysis of blood plasma samples after induced glycemia : in this case, a glucose calibration is necessary for each set of 3–4 blood additions. The dotted line, representing the second derivative of the current  $I_1$ – $I_2$ , has been successfully used for monitoring a printing device of the peak of the first derivative, *i.e.* the dynamic response.<sup>7</sup>

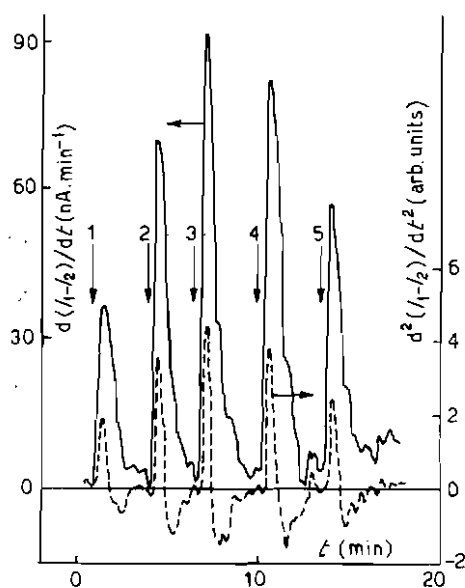


Fig. 3.  
Induced glycemia. (—) first and (---) second derivative of the current after injection of 50 mm<sup>3</sup> (μl) non deproteinized plasma samples. to 20 cm<sup>3</sup> buffer (1) calibration of the electrode with a 10 μM glucose pulse, (2–5) glucose determination in plasma samples taken after 0 (2), 30 (3), 60 (4) and 120 minutes (5) after induced glycemia. Corresponding glucose content were respectively 1.1, 1.7, 1.4 and 1.0 g/l.

The glucose electrode may be used in a large temperature range, from 15 to 40 °C. As both responses are very sensitive to temperature (about 4–5 % / °C at 30 °C) it is necessary to carefully thermostate the solutions in contact with both electrodes.

## Conclusions

Industrially reconstituted collagen films were found suitable for glucose oxidase immobilization; the enzymatic activity was maintained and its stability enhanced. A membrane loading of 50 to 100 mU per membrane (1 cm diameter) was sufficient to obtain a very sensitive glucose electrode when associated with amperometric hydrogen peroxide detection. Stationary and dynamic responses of this glucose electrode was proportionnal to glucose concentration from  $3-5 \times 10^{-8}$  to  $3-5 \times 10^{-3}$  M. Furthermore, this sensor was used for whole blood samples analysis in induced glycemia.

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## References

- <sup>1</sup> L.C. CLARK and C. LYONS, *Ann. N.Y. Acad. Sci.* **102**, 29 (1962)
- <sup>2</sup> G.G. GUILBAULT, in *Immobilized Enzymes, Antigens, Antibodies and Peptides*, H.H. WEETALL (Editor), Marcel Dekker Inc., New York (1975) p. 293
- <sup>3</sup> P.R. COULET, J.H. JULLIARD, D.C. GAUTHERON, Brevet d'Invention Français (ANVAR) n° 2.235.133 (1973)
- <sup>4</sup> P.R. COULET, J. H. JULLIARD and D.C. GAUTHERON, *Biotechnol. Bioeng.* **16**, 1055 (1974)
- <sup>5</sup> P.R. COULET and D.C. GAUTHERON, in *Analysis and Control of Immobilized Enzyme Systems*, D. THOMAS and J.P. KERNEVEZ (Editors), North Holland, Amsterdam (1976) p. 165
- <sup>6</sup> D.R. THEVENOT, P.R. COULET and D.C. GAUTHERON, in *Enzyme Engineering* Plenum Press, New York (1978) Vol. 4, p. 221
- <sup>7</sup> D.R. THEVENOT, P.R. COULET, R. STERNBERG, J. LAURENT and D.G. GAUTHERON to be published